plant species including maize (GenBank Accession No. D73410), rice (Accession No. D73411), black-eyed pea (Accession No. U92656), *Pimpinella brachycarpa* (Accession No. U96438) and *A. thaliana* (GenBank Accession No.U84568 and GenBank Accession No. AF027402) (Dyer *et al.,* 1995; Pappan *et al.,* 1997a; 1997b; Qin *et al.,* 1997; Ueki *et al.,* 1995, Morioka *et al.,* 1997). Three distinct PLD cDNAs were identified from *A. thalania* and designated PLD α, β and γ (Dyer *et al.,* 1995, Pappan *et al.,* 1997a; 1997b).

There is a 73-90% amino acid sequence identity among the PLD α cDNAs from castor bean, rice, maize and *Arabidopsis*. This is in contrast to the *A. thalania* PLD α protein having only 40% identity to the *A. thalania* PLD β and γ, yet, PLD β and γ were reported to have 66% identity to each other at the amino acid sequence level (Pappan and Wang, 1998). It has been reported that PLD β is more closely related to the proteins cloned from yeast and humans than the α form (Wang, 1997). The *A. thalania* PLDα has a molecular mass of 91,800 daltons whereas the *A. thalania* β and γ reported molecular mass is 109,000 and 95,500 daltons, respectively (Pappan and Wang, 1998). Alignments of the plant PLD sequences have revealed three conserved regions. A calcium phospholipid-binding domain (C2) was present in all plant PLDs (but is lacking in all mammalian and yeast PLDs to date) near the N-terminus of the sequence. Second, two putative catalytic HxKxxxD (SEQ ID NO:15) motifs have been identified in all PLDs cloned from plant, animals and yeast. It has been hypothesized that the absolute conservation of His, Lys and Asp residues at these positions suggest these residues are in the active site (Pointing and Kerr, 1996; Sung, 1997). Third, a binding site for PIP_2 also was identified surrounding the second HKD motif. This region is rich in basic residues and has been reported to be responsible for polyphosphoinositide binding in proteins such as gelsolin Ph and villin (Divecha and Irvine, 1995).

4.6 Role of PLD in Plants

Historically phospholipase D activity has been associated with large-scale membrane degradation of lipids during germination and senescence (Munnik *et al.,* 1998). However, more recent studies suggest that in addition to membrane degradation, PLD may also have a more highly regulated role involving signal transduction (Munnik *et al.,* 1995; Ryu and Wang, 1996, Nakamura, 1996; Ritchie and Gilroy, 1998). High activity of the PLD

TABLE 1

DEGENERATE OLIGONUCLEOTIDE PRIMERS FOR PCR™ AND RT-PCR™ STUDIES

Primer	Amino acid sequence[a] Nucleotide sequence[b]	Length (nt)	T_m[c] (°C)	Degeneracy (n-fold)
-[d]1	GgQHKTIEMM (SEQ ID NO:3) 5'-catcatytcdatngtyttrtgytgcc-3' (SEQ ID NO:4)	26	70	192
+2	IYTHHEKac (SEQ ID NO:5) 5' athtayacncaycaygaraarac3' (SEQ ID NO:6)	23	54	384
-3	CnIYTHHEKac (SEQ ID NO:7) 5'-gtyttytcrtgrtgngtrtadatng3' (SEQ ID NO:8)	25	63	1536
+4	ECWFWCgg (SEQ ID NO:9) 5' gartgytggttytggtgygg3' (SEQ ID NO:10)	20	67	16
+5	HGKCWEDM (SEQ ID NO:11) 5' cayggnaartgytgggargayatg3' (SEQ ID NO:12)	24	68	128
-6	EEPENMECg (SEQ ID NO:13) 5' crcaytccatrttytcnggytcytc3' (SEQ ID NO:14)	25	69	256

[a]Amino acid sequences are capitalized.

[b]Nucleotide sequences are in lower case. Symbols used to denote multiple sequences are as follows: y=C or T; r=A or G; d=G or A or T; n=A or C or G or T.

[c]T_m's were calculated using "DNA Synthesis Oligo Calculator" (http://www.biotech.ufl.edu/cgi-bin/doa.cgi/).

[d]The + and – signs indicate the coding strand or complimentary to the coding strand, respectively.

5.2.11 SUBCLONING OF PCR™ PRODUCTS

PCR™ products (in 5 µl) were reamplified in a 50 µl reaction mixture containing 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100™, 10 µg/ml nuclease-free BSA, 2.5 units of *Pfu* DNA polymerase (Stratagene), 0.2 mM total of dATP, dTTP, dGTP, dCTP and 0.5 µM of the same primers used in original amplification. The reaction was incubated for 45 sec at 95°C, then followed by 35 cycles of amplification with 30 sec at 95°C, 30 sec at 55°C to 65°C (annealing temperature was identical to original PCR™ amplification), 2.5 min at 72°C. After the last cycle, the amplification was extended for 10 min at 72°C. The PCR™ reaction product was immediately purified from the reaction mixture using the Prep-A-Gene™ DNA purification kit. An aliquot (5 µl) was quantified in a 3% agarose gel with DNA molecular mass markers (10-200 ng/band, corresponding to 100-

5.5 EXAMPLE 5 - ISOLATION AND DNA SEQUENCE OF A PLD β GENE IN TOBACCO

To better understand the role of PLD in NAPE metabolism, molecular analysis was performed to determine if PLD β or γ were present in tobacco cell suspensions. A pair of degenerate PCR™ primers were designed based on the amino acid sequences of the *A. thalania* PLD β (Pappan *et al.,* 1997a; 1997b) and PLD γ (Qin *et al.,* 1997) gene products. A cDNA library was constructed from mRNA isolated from a *Nicotiana tabacum* NT-1 cell line in early exponential growth phase (obtained from Dr. G. An, Washington State University, Pullman, WA). A cDNA fragment was amplified from the tobacco cDNA library with a degenerate primer combination (Table 1, Primer -1 and +5) using PCR™. The PCR™ fragment was approximately 1.2 kb. Subcloning was performed for sequencing analysis using pZErO-2.1™ as the vector. Recombinant clones in *E. coli* Top 10™ cells were obtained and four were randomly chosen for plasmid DNA isolation and restriction digestion (FIG. 8). Vector DNA was digested with restriction enzymes *Sac*I and *Xba*I. An insert was detected in two of the samples, designated "clone II1" and "clone II6" (see FIG. 8, lanes 10 and 13). The sequence of clone II6 was identical to that of clone II1. To completely characterize the PCR™ product, clone II1 was digested with enzymes at the multiple cloning site of the pZErO-2.1™ vector. Six different enzymes were incubated with clone II1 and electrophoresed in a 1.5% agarose gel (FIG. 9). *Hin*dIII cleaved the PCR™ fragment at an internal site (FIG. 9. lane 2). Both the small and the large fragment of clone II1 from *Hin*dIII digestion were subcloned and sequenced. FIG. 10 shows the physical map of clone II1 showing the internal restriction site for *Hin*dIII, the multiple cloning site, and the direction of the M13 forward and reverse primers used in the sequencing reactions.

Both strands of the cDNA fragment in the pZErO-2.1™ vector designated clone II1 and the subfragment thereof were sequenced using M13 forward and reverse primers. The cDNA fragment was 1170 bp. The deduced amino acid sequence is shown below the nucleotide sequence in FIG. 11. The degenerate primers used to generate the sequence were found on the 5'- and the 3'-end of the fragment, as indicated by the dashed arrow lines. Included within this 390 amino acid segment was one putative catalytic domain, denoted HxKxxxxD (SEQ ID NO:15). Using the BLAST program (Altschul *et al.,* 1990) clone II1 had highest homology to *A. thalania* PLD β (GenBank Accession No. U84568), followed by the *A. thalania* PLD γ (GenBank Accession No. KFO2408) over the length of the 1.2-kb fragment.